The L45 loop in type I receptors for TGF-β family members is a critical determinant in specifying Smad isoform activation

Urban Persson[1,a], Hiroto Izumi[1,a], Serhiy Souchelnytskyi[b], Susumu Itoh[b], Susanne Grimsby[b], Ulla Engström[b], Carl-Henrik Heldin[b], Keiko Funa[a], Peter ten Dijke[b,*]

[a]*Department of Anatomy and Cell Biology, Göteborg University, Box 420, SE-405 30 Göteborg, Sweden*
[b]*Ludwig Institute for Cancer Research, Box 595, SE-751 24 Uppsala, Sweden*

Received 16 July 1998; revised version received 31 July 1998

Abstract Transforming growth factor-β (TGF-β) and bone morphogenetic proteins (BMPs) signal via distinct type I and type II receptors and Smad proteins. A nine amino acid sequence between kinase subdomains IV and V in type I receptors, termed the L45 loop, has been shown to be important in conferring signalling specificity. We examined the responses of a mutant TGF-β type I receptor (TβR-I) and a mutant BMPR-IB, in which the L45 regions of these two receptors were exchanged. Swapping the four amino acid residues that are different in BMPR-IB for those in TβR-I, and vice versa, switched their type I receptor-restricted Smad activation and specificity in transcriptional responses. These studies identify the L45 loop regions in type I receptors as critical determinants in specifying Smad isoform activation.

Key words: Bone morphogenetic protein; Receptor specificity; Signal transduction; Smad; Transforming growth factor-β

1. Introduction

Transforming growth factor-βs (TGF-βs), activins, müllerian inhibiting substance (MIS) and bone morphogenetic proteins (BMPs) are structurally related proteins that control a broad range of cellular processes, including proliferation, differentiation, apoptosis and migration. Although certain biological activities are shared between multiple ligands, each ligand has also distinct biological responses [1–4]. Signalling of these pleiotropic dimeric molecules occurs via ligand-induced complex formation of two related serine/threonine kinase receptors, i.e. type I and type II receptor [5–7]. Different TGF-β family members bind distinct type II and type I receptors. Seven type I receptors, also termed activin receptor-like kinases (ALKs) have been identified to date; ALK5 is a TGF-β type I receptor (TβR-I), ALK2 and ALK4 are known as activin type I receptor (ActR-I) and ActR-IB, respectively, and ALK3 and ALK6 as BMP type IA receptor (BMPR-IA) and BMPR-IB, respectively. The physiological ligands for ALK1 and ALK7 are unknown [5–7]. Both receptors have been shown to be essential for signalling. Type I receptors act downstream of type II receptors; type I receptor kinases become activated upon phosphorylation by the constitutively active type II receptor kinase and propagate the signal downstream [8,9]. Consistent with these findings, type I receptors were found to determine the specificity of the intracellular signals induced by different TGF-β family members [10,11]. Using chimeric receptors of ALK2 and ALK5 the L45 region was found to be important in determining the signalling specificity of type I receptors [12].

Members of the Smad family of proteins have been shown to play a pivotal role in the intracellular signalling of TGF-β family members [13–16]. The so-called pathway-restricted Smads interact with and become phosphorylated by the type I receptor kinases at two serine residues in their extreme C-terminal ends [15,17–27], whereafter they form heteromeric complexes with common-mediator Smad4 [22,28,29]. The heteromeric complex is then translocated to the nucleus [15,17,20,24] and controls the transcription of target genes [30]. Smad proteins have two regions of high sequence similarity at their N- and C-terminal regions, termed Mad homology (MH)1 and MH2 domain, respectively. The N-terminal region has been shown to have potential of direct sequence specific DNA binding activity [31–35], and the C-terminal domain can act as a transcriptional activator [17,36].

Different pathway-restricted Smads couple with different type I receptors and thus specify their responses; whereas ALK5 interacts with Smad2 and Smad3, but not Smad1 or Smad5. ALK6 binds to Smad1 and Smad5, but not Smad2 or Smad3 [15,20–24,37,38]. In the present investigation we have investigated whether exchanging the L45 loop regions between ALK5 and ALK6 is sufficient to switch their specificity in Smad activation and Smad-mediated transcriptional responses.

2. Materials and methods

2.1. Expression plasmids

Expression constructs for wild-type and constitutively active receptors have been described [24]. ALK5BL45 and ALK6TL45 were made by polymerase chain reaction (PCR)-mediated approach using a site-directed mutagenesis kit (Stratagene) and subcloning in pCDNA3. To obtain the constitutively active versions of ALK5BL45 and ALK6TL45 the L45 regions were subcloned into the constitutively active versions of ALK5 and ALK6, respectively. GAL4-Lux was obtained from Johan Ericsson [39]. pSG424, pSG424Smad1(FL) and pSG424Smad2(FL) have been described [17]. $(CAGA)_{12}$MLP-Luc, a multimerised response element derived from the PAI-1 promoter and inducible by TGF-β, but not BMP, and $(SBE)_4$-Lux, a multimerised response element derived from the JunB promoter and inducible by both TGF-β and BMP, have been described [34,35].

2.2. Antisera

For generation of antiserum PS2 recognising the phosphorylated C-tail in Smad2, a peptide KKK-SSpMSp, containing two C-terminal phosphoserine residues, was coupled to keyhole limpet hemocyanin

*Corresponding author. Fax: (46) (18) 160420.
E-mail: Peter.ten_Dijke@LICR.uu.se

[1]These authors contributed equally to this work.

PII: S0014-5793(98)00954-5

(KLH) using glutaraldehyde, mixed with Freund's adjuvant, and used to immunise rabbits. In a similar manner PS1, recognising the phosphorylated C-tail in Smad1 was generated; a peptide KKK-NPISSVS, containing two C-terminal phosphoserine residues was coupled to KLH. The N-terminal lysine residues were added to facilitate coupling to KLH. Specificity of the antiserum was tested after immunoprecipitation of metabolically labelled cell lysates from COS cells transfected with Smads in the absence or presence of activated type I receptors, as well as from non-transfected cells before and after stimulation with TGF-β1 and BMP-7 (data not shown). Anti-Flag antibody was obtained from Kodak, and haemagglutinin (HA) antibody, also termed YPY, was raised in rabbits against a peptide containing two HA epitopes coupled to KLH.

2.3. Cell assays

Transfection of cells, metabolic labelling, immunoprecipitation, affinity cross-linking, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were performed as described [24].

2.4. Transcriptional response assay

Mv1Lu cells lacking functional TβR-I (termed R-mutant) [40] were transfected with reporter constructs using Superfect (Qiagen). In each experiment, equal amounts of DNA were transfected. Luciferase activity was measured as described [24], and normalised for transfection efficiency. Results shown are representative of at least three independent experiments.

3. Results and discussion

3.1. TβR-I/ALK5 and BMPR-IB/ALK6 with exchanged L45 loop regions

To determine whether the L45 loop can define receptor-Smad interactions, we compared the ability of ALK5-restricted Smads and ALK6-restricted Smads to interact with mutants of ALK5 and ALK6, in which the L45 loops were exchanged, termed ALK5BL45 and ALK6TL45, respectively. Mutant receptors were made using a PCR-based approach. The L45 loop in ALK5, ADNKDNGTW, differs in four amino acid residues from the L45 loop in ALK6, ADIKGTGSW (Fig. 1). Both mutant receptors were found to bind ligand and

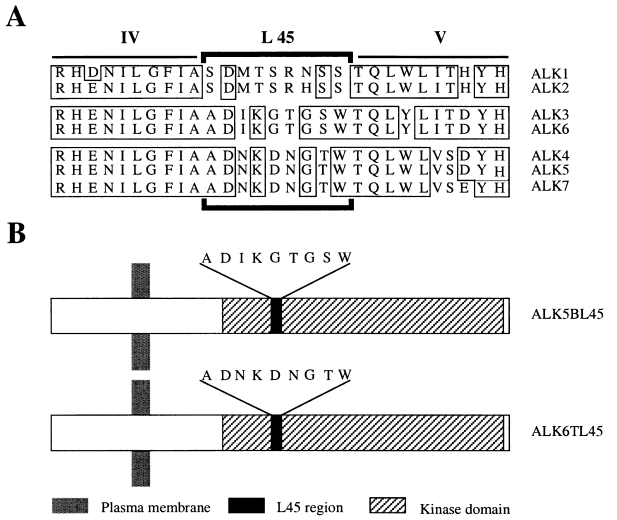

Fig. 1. A: Amino acid sequence alignment of the L45 loops from the type I receptors. The type I receptors are divided in three subgroups based upon their amino acid sequence similarities within the L45 loop. Parts of the flanking kinase subdomains IV and V are also shown. Amino acid residues that are conserved in more than 4 receptors are boxed. ALK-2, ALK3, ALK4, ALK5 and ALK6 are also known as ActR-I, BMPR-IA, ActR-IB, TβR-I and BMPR-IB, respectively. B: Schematic overview of ALK5BL45 and ALK6TL45.

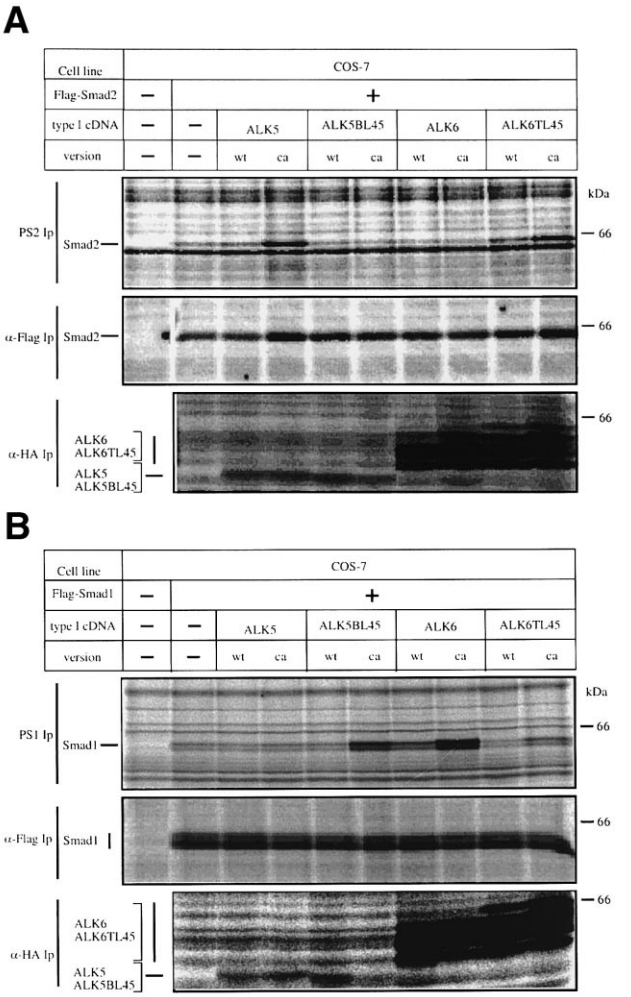

Fig. 2. Smad phosphorylation by receptors. Comparison of wild-type vs. constitutively active versions of ALK5 (TβR-I)-, ALK6 (BMPR-IB)-, ALK5BL45- and ALK6TL45-mediated phosphorylation of (A) Smad2 and (B) Smad1. Phosphorylation levels of Smad2 and Smad1 were examined on metabolically labeled cultures using PS2 and PS1 antiserum, respectively. Expression of HA-tagged type I receptors and Flag-tagged Smads were determined by immunoprecipitation with HA and Flag antiserum, respectively, of parallel cultures which were metabolically labelled. wt, wild-type; ca, constitutively active.

had an intrinsic kinase activity comparable to that of the wild-type counterparts (data not shown).

3.2. Switching receptor-restricted Smad phosphorylation

Smad2 and Smad1 become phosphorylated upon interaction with activated ALK5 and activated ALK6, respectively [15,20–24,37,38]. We therefore compared the ability of ALK5, ALK6, ALK6TL45 and ALK5BL45 to phosphorylate these two Smads. COS cells were transfected with wild-type (wt) or constitutively active (ca) HA-tagged type I receptors together with Flag-tagged Smad2. The phosphorylation levels of Smad2 were examined by ^{35}S metabolic labelling followed by immunoprecipitation of the cell lysates using PS2 antiserum that specifically recognises the phosphorylated C-tail of Smad2. As expected, constitutively active ALK5, but not constitutively active ALK6, phosphorylated Smad2 at serine residues in its C-tail. Interestingly, constitutively active

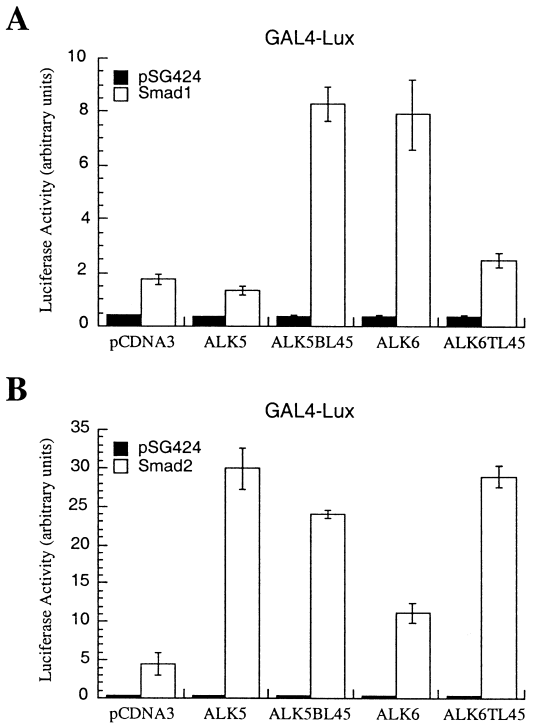

Fig. 3. Stimulation of transcriptional activity of Smad1 and Smad2 by receptors. Empty pSG424 vector, (A) pSG424Smad1(FL) or (B) pSG424Smad2(FL) constructs were transfected into R-mutant cells together with constitutively active versions of ALK5, ALK6, ALK5BL45 or ALK6TL45, and a GAL4-Lux reporter gene. Luciferase activity was normalised for transfection efficiency. Data are the means ± S.D. of triplicate experiments.
←

ALK6TL45 gained ability to phosphorylate Smad2, whereas constitutively active ALK5BL45 failed to phosphorylate Smad2 (Fig. 2A). In an analogous manner the Smad1 phosphorylation by ALK5, ALK6, ALK6TL45 and ALK5BL45 was examined in transfected COS cells. The phosphorylation levels of Smad1 were examined by ^{35}S metabolic labelling followed by immunoprecipitation of the cell lysates using PS1 antiserum that specifically recognises the phosphorylated C-tail of Smad1. Constitutively active ALK5BL45, but not constitutively active ALK5, phosphorylated Smad1, and the level of Smad1 phosphorylation induced by ALK6TL45 was much lower than by constitutively active ALK6 (Fig. 2B). Thus, swapping the 4 different amino acid residues in L45 loop regions enabled ALK6 to phosphorylate Smad2 and ALK5 to phosphorylate Smad1.

3.3. Switching receptor-restricted Smad transcriptional activation

Smads were shown to have transcriptional activity; when fused to GAL4 DNA binding domain, Smad1 and Smad2 activate a luciferase reporter containing GAL4 DNA binding sites upon activation by BMP and TGF-β, respectively [17,36]. We assayed the ability of constitutively active derivatives of ALK5, ALK6, ALK5BL45 or ALK6TL45 to activate GAL4-Smad1- or GAL4-Smad2-mediated reporter activities in Mv1Lu mink epithelial cells lacking functional ALK5, termed

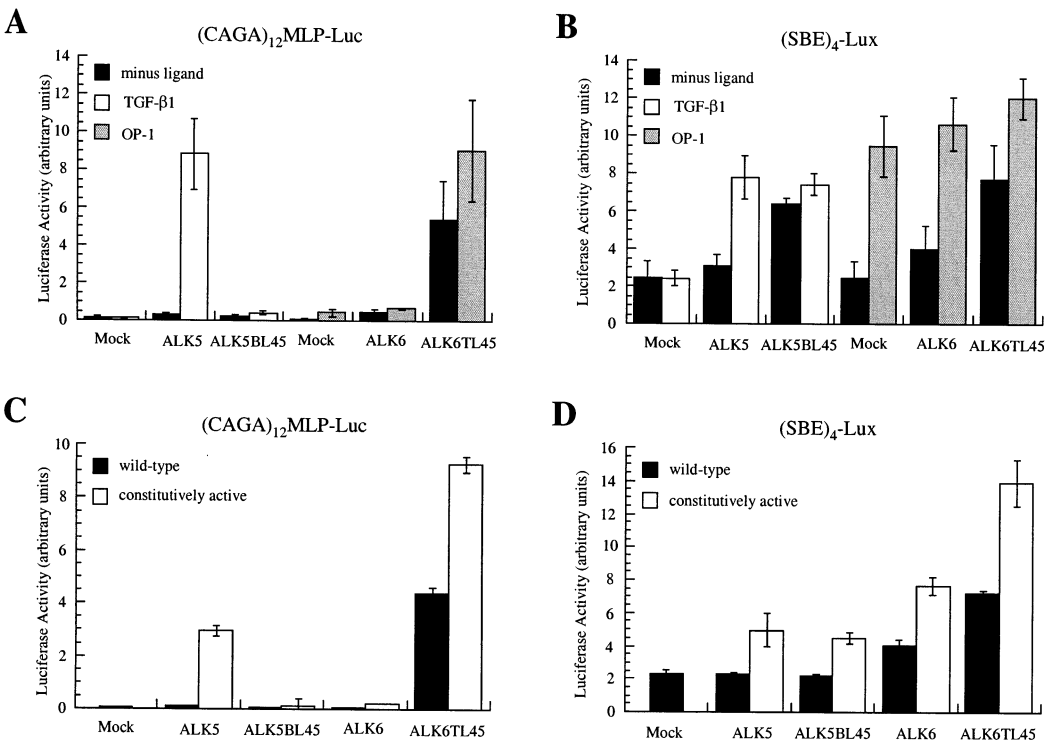

Fig. 4. Transcriptional effects of receptors. Receptor constructs were transfected into R mutant cells together with (A) $(CAGA)_{12}$MLP-Luc or (B) $(SBE)_4$-Lux reporters, and after stimulation with ligand luciferase activity was measured. In a similar way constitutively active and wild-type receptor constructs were transfected into R-mutant cells together with (C) $(CAGA)_{12}$MLP-Luc or (D) $(SBE)_4$-Lux reporters, and luciferase activity was measured. Data are the means ± S.D. of triplicate experiments.

R-mutant cells (Fig. 3). We found that ALK5BL45 mimicked ALK6 since both activated GAL4-Smad1. In addition, the ability to activate GAL4-Smad1 by ALK6TL45 was much weaker than that by ALK6. Moreover, ALK6TL45 and ALK5 shared a similar ability to activate GAL4-Smad2. However, ALK5BL45 was able to transcriptionally activate Smad2, although less efficiently than ALK5. Thus, swapping of the L45 loop regions between ALK5 and ALK6 was sufficient to obtain a near to complete switch in their specificity of Smad activation, indicating that the L45 loop structural motif has a critical role in specifying which Smads become activated.

3.4. Switching receptor-restricted transcriptional responses

We have previously described a transcriptional reporter $(CAGA)_{12}$MLP-Luc containing response elements derived from the PAI-1 promoter, which is inducible by TGF-β but not BMPs [34]. In addition, we have shown that a transcriptional $(SBE)_4$-Lux reporter with response elements derived from the *JunB* promoter, can be efficiently induced by TGF-β as well as BMPs [35]. Using these two reporter constructs we analysed the ligand mediated transcriptional responses upon activation with ALK5, ALK6, ALK5BL45 or ALK6TL45 in R-mutant cells. In order to circumvent the possibility of ligand induced signalling complexes with endogenous receptors we also analysed wild-type receptors vs. their constitutively active counterparts (Fig. 4). The $(CAGA)_{12}$MLP-Luc reporter was activated with ALK6TL45 as well as with ALK5, whereas ALK5BL45 like ALK6 did not activate this. As expected, all constructs activated the $(SBE)_4$-Lux reporter, which does not discriminate between ALK5- and ALK6-induced responses. The reason for the relatively high level of ligand-independent signalling activity of ALK6TL45, and to a lesser extent ALK5BL45, compared to their wild-type counterparts is unknown. Thus, exchanging the L45 loop between ALK5 and ALK6 altered their ability to activate transcriptional responses, in accordance with their changed specificity in Smad isoform activation.

3.5. Concluding remarks

The L45 loop structural motif in type I receptors specifies Smad isoform activation. Our results are consistent with a recent report by Chen et al. [41]; they also find that L45 loop regions establish the specificity of receptor-Smad interactions. The L45 loop regions of type I receptors can be placed in three distinct subgroups (Fig. 1); ALK4, ALK5 and ALK7 have an identical L45 loop amino acid sequence, as have ALK3 and ALK6, and there is only one amino acid difference between the L45 loops of ALK1 and ALK2 (Fig. 1). It is possible that the interactions of type I receptors within one subgroup with different Smad proteins is similar.

Acknowledgements: We are grateful to Joan Massagué, Napoleone Ferrara, Jean-Michel Gauthier, Johan Ericsson and Kuber Sampath for reagents. U.P., H.I. and K.F. were supported by the Swedish Medical Research Councils, the Swedish Cancer Society and the Swedish Foundation for International Cooperation in Research and Higher Education (STINT). A visiting fellowship to H.I. was provided through the Swedish Cancer Society. In addition, this work was supported by the IngaBritt and Arne Lundberg Foundation and by a grant from the European Commission, Trans Mobility Researchers network (contract no. ERBFMRXCT980216).

References

[1] Roberts, A.B. and Sporn, M.B. (1990) in: Peptide Growth Factors and Their Receptors, Part I, Vol. 95 (Sporn, M.B. and Roberts, A.B., Eds.) pp. 419–472, Springer-Verlag, Berlin.
[2] Vale, W., Hsueh, A., Rivier, C. and Yu, J. (1990) in: Peptide Growth Factors and Their Receptors, Part II (Sporn, M.B. and Roberts, A.B., Eds.) pp. 211–248, Springer-Verlag, Berlin.
[3] Kingsley, D.M. (1994) Genes Dev. 8, 133–146.
[4] Reddi, A.H. (1997) Cytokine Growth Factor Rev. 8, 11–20.
[5] Massagué, J., Hata, A. and Liu, F. (1997) Trends Cell Biol. 7, 187–192.
[6] Derynck, R. and Feng, X.H. (1997) Biochim. Biophys. Acta Rev. Cancer 1333, F105–F150.
[7] Heldin, C.-H., Miyazono, K. and ten Dijke, P. (1997) Nature 390, 465–471.
[8] Wrana, J.L., Attisano, L., Wieser, R., Ventura, F. and Massagué, J. (1994) Nature 370, 341–347.
[9] Wieser, R., Wrana, J.L. and Massagué, J. (1995) EMBO J. 14, 2199–2208.
[10] Cárcamo, J., Weis, F., Ventura, F., Wieser, R., Wrana, J.L., Attisano, L. and Massagué, J. (1994) Mol. Cell. Biol. 14, 3810–3821.
[11] Persson, U., Souchelnytskyi, S., Franzén, P., Miyazono, K., ten Dijke, P. and Heldin, C.-H. (1997) J. Biol. Chem. 272, 21187–21194.
[12] Feng, X.-H. and Derynck, R. (1997) EMBO J. 16, 3912–3923.
[13] Sekelsky, J.J., Newfeld, S.J., Raftery, L.A., Chartoff, E.H. and Gelbart, W.M. (1995) Genetics 139, 1347–1358.
[14] Savage, C., Das, P., Finelli, A.L., Townsend, S.R., Sun, C.-Y., Baird, S.E. and Padgett, R.W. (1996) Proc. Natl. Acad. Sci. USA 93, 790–794.
[15] Hoodless, P.A., Haerry, T., Abdollah, S., Stapleton, M., O'Connor, M.B., Attisano, L. and Wrana, J.L. (1996) Cell 85, 489–500.
[16] Graff, J.M., Bansal, A. and Melton, D.A. (1996) Cell 85, 479–487.
[17] Liu, F., Hata, A., Baker, J.C., Doody, J., Cárcamo, J., Harland, R.M. and Massagué, J. (1996) Nature 381, 620–623.
[18] Lechleider, R.J., de Caestecker, M.P., Dehejia, A., Polymeropoulos, M.H. and Roberts, A.B. (1996) J. Biol. Chem. 271, 17617–17620.
[19] Yingling, J.M., Das, P., Savage, C., Zhang, M., Padgett, R.W. and Wang, X.-F. (1996) Proc. Natl. Acad. Sci. USA 93, 8940–8944.
[20] Eppert, K. et al. (1996) Cell 86, 543–552.
[21] Zhang, Y., Feng, X.-H., Wu, R.-Y. and Derynck, R. (1996) Nature 383, 168–172.
[22] Lagna, G., Hata, A., Hemmati-Brivanlou, A. and Massagué, J. (1996) Nature 383, 832–836.
[23] Macías-Silva, M., Abdollah, S., Hoodless, P.A., Pirone, R., Attisano, L. and Wrana, J.L. (1996) Cell 87, 1215–1224.
[24] Nakao, A. et al. (1997) EMBO J. 16, 5353–5362.
[25] Kretzschmar, M., Liu, F., Hata, A., Doody, J. and Massagué, J. (1997) Genes Dev. 11, 984–995.
[26] Abdollah, S., Macías-Silva, M., Tsukazaki, T., Hayashi, H., Attisano, L. and Wrana, J.L. (1997) J. Biol. Chem. 272, 27678–27685.
[27] Souchelnytskyi, S., Tamaki, K., Engström, U., Wernstedt, C., ten Dijke, P. and Heldin, C.-H. (1997) J. Biol. Chem. 272, 28107–28115.
[28] Wu, R.-Y., Zhang, Y., Feng, X.-H. and Derynck, R. (1997) Mol. Cell. Biol. 17, 2521–2528.
[29] Zhang, Y., Musci, T. and Derynck, R. (1997) Curr. Biol. 7, 270–276.
[30] Chen, X., Rubock, M.J. and Whitman, M. (1996) Nature 383, 691–696.
[31] Kim, J., Johnson, K., Chen, H.J., Carroll, S. and Laughon, A. (1997) Nature 388, 304–308.
[32] Yingling, J.M., Datto, M.B., Wong, C., Frederick, J.P., Liberati, N.T. and Wang, X.F. (1997) Mol. Cell. Biol. 17, 7019–7028.
[33] Zawel, L., Dai, J.L., Buckhaults, P., Zhou, S., Kinzler, K.W., Vogelstein, B. and Kern, S.E. (1998) Mol. Cell 1, 611–617.
[34] Dennler, S., Itoh, S., Vivien, D., ten Dijke, P., Huet, S. and Gauthier, J.-M. (1998) EMBO J. 17, 3091–3100.

[35] Jonk, L.J.C., Itoh, S., Heldin, C.-H., ten Dijke, P. and Kruijer, W. (1998) J. Biol. Chem., in press.
[36] Meersseman, G. et al. (1997) Mech. Dev. 61, 127–140.
[37] Nishimura, R., Kato, Y., Chen, D., Harris, S.E., Mundy, G.R. and Yoneda, T. (1998) J. Biol. Chem. 273, 1872–1879.
[38] Tamaki, K., Souchelnytskyi, S., Itoh, S., Nakao, A., Sampath, K., Heldin, C.-H. and ten Dijke, P. (1998) J. Cell Phys., in press.
[39] Ericsson, J. and Edwards, P.A. (1998) J. Biol. Chem., in press.
[40] Laiho, M., Weis, F.M.B. and Massagué, J. (1990) J. Biol. Chem. 265, 18518–18524.
[41] Chen, Y.-G., Hata, A., Lo, R.S., Wotton, D., Shi, Y., Pavletich, N. and Massagué, J. (1998) Genes Dev. 12, 2144–2152.